

Structural and Spatial Determinants Regulating TC21 Activation by RasGRF Family Nucleotide Exchange Factors

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RasGRF family guanine nucleotide exchange factors (GEFs) promote guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange on several Ras GTPases, including H-Ras and TC21. Although the mechanisms controlling RasGRF function as an H-Ras exchange factor are relatively well characterized, little is known about how TC21 activation is regulated. Here, we have studied the structural and spatial requirements involved in RasGRF 1/2 exchange activity on TC21. We show that RasGRF GEFs can activate TC21 in all of its sublocalizations except at the Golgi complex. We also demonstrate that TC21 susceptibility to activation by RasGRF GEFs depends on its posttranslational modifications: farnesylated TC21 can be activated by both RasGRF1 and RasGRF2, whereas geranylgeranylated TC21 is unresponsive to RasGRF2. Importantly, we show that RasGRF GEFs ability to catalyze exchange on farnesylated TC21 resides in its pleckstrin homology 1 domain, by a mechanism independent of localization and of its ability to associate to membranes. Finally, our data indicate that Cdc42-GDP can inhibit TC21 activation by RasGRF GEFs, demonstrating that Cdc42 negatively affects the functions of RasGRF GEFs irrespective of the GTPase being targeted.

INTRODUCTION

Ras family GTPases regulate key cellular processes, including proliferation, differentiation, and cell survival (Takai *et al.*, 2001), and their constitutive activation, resulting from point mutations, underlies severe pathologies such as cancer. Apart from the classical Ras proteins: H-Ras, N-Ras, and K-Ras, another family member frequently found mutated in human cancers is TC21 (Chan *et al.*, 1994; Huang *et al.*, 1995; Barker and Crompton, 1998). TC21 belongs to the R-Ras subfamily and displays an overall 55% homology with the classical Ras proteins, including identical core effector regions (Drivas *et al.*, 1990). As a consequence, TC21 shares most effectors with the classical Ras proteins. These include Raf (Movilla *et al.*, 1999; Rosario *et al.*, 1999), phosphatidylinositol 3-kinase (Rosario *et al.*, 2001; Murphy *et al.*, 2002), Ral-GDS (Lopez-Barahona *et al.*, 1996; Rosario *et al.*, 2001), and phospholipase C γ (Kelley *et al.*, 2004). In contrast, TC21 possesses a unique C-terminal CAAX box, which determines that TC21 can be both farnesylated and geranylgeranylated (Carboni *et al.*, 1995; Roskoski and Ritchie, 1998; Reid *et al.*, 2004), unlike the classical Ras proteins that are farnesylated (Seabra, 1998). In addition, and also unlike Ras proteins, the TC21 C terminus does not harbor a polybasic sequence nor it is clear still whether it can be palmitoylated like R-Ras (Furuhjelm and Peranen, 2003). These divergences in the nature of the anchors by which small GTPases associate to different membrane systems and microdomains (Rocks *et al.*, 2006) may result in TC21 subcellular distribution being dif-

ferent from that exhibited by Ras, an aspect of TC21 biology yet unknown.

Ras GTPases cycle between guanosine diphosphate (GDP)-bound inactive states and guanosine triphosphate (GTP)-bound active states. The transit from inactive to active is catalyzed by proteins known as guanine nucleotide exchange factors (GEFs). TC21 and Ras become activated by similar stimuli. Concomitantly, they share several GEFs: those belonging to the RasGRF and RasGRP but not to the SOS families (Ohba *et al.*, 2000). The RasGRF family includes RasGRF1 and RasGRF2, which display a 75% homology (Fam *et al.*, 1997; Fan *et al.*, 1998; de Hoog *et al.*, 2000). The primary structures of these GEFs reveal several motifs presumably involved in diverse regulatory mechanisms. These include a Dbl homology (DH) domain, mainly present in GEFs for Rho family GTPases flanked by two pleckstrin homology (PH) domains of largely unknown function, although suggested to play a role in targeting mechanisms (Schmidt and Hall, 2002). Interestingly, whereas RasGRF2 does not require its DH domain to activate Ras (de Hoog *et al.*, 2000), RasGRF1 does (Arozarena *et al.*, 2000). Despite their high degree of homology, other functional differences have been reported between RasGRF1 and -2 with respect to their specificity toward cognate GTPases. Contrarily to RasGRF1, RasGRF2 cannot activate R-Ras (Gotoh *et al.*, 2001). Alternatively, RasGRF2 promotes nucleotide exchange on H-, N-, and K-RasB in vivo (de Hoog *et al.*, 2000; Matallanas *et al.*, 2003), whereas RasGRF1 only activates H-Ras (Jones and Jackson, 1998; Matallanas *et al.*, 2003). Thus far, it is not known whether, like RasGRF1 (Ohba *et al.*, 2000), RasGRF2 can activate TC21.

Regarding their regulation, RasGRF1 and -2 are mainly activated by G protein-coupled receptors and by some tyrosine kinase receptors (Shou *et al.*, 1995; Mattingly and Macara, 1996; Zippel *et al.*, 1996; Fam *et al.*, 1997; Krapivinsky *et*

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al., 2003; Tian *et al.*, 2004; Robinson *et al.*, 2005). RasGRFs are also particularly sensitive to changes in calcium levels, by a mechanism mediated through a calmodulin-binding isoleucine-glutamine (IQ) domain present in the N terminus of these GEFs (Farnsworth *et al.*, 1995; Buchsbaum *et al.*, 1996; Fam *et al.*, 1997; Fan *et al.*, 1998). Furthermore, we have demonstrated that RasGRFs competence to activate Ras is regulated by the Rho family GTPase Cdc42, by yet unknown mechanisms that require a functional DH domain (Arozarena *et al.*, 2000, 2001). In addition, RasGRF GEFs exhibit a marked spatial specificity toward pools of Ras present at distinct membrane systems, being active on Ras at the plasma membrane and at the endoplasmic reticulum but not at the Golgi complex (Arozarena *et al.*, 2004).

In this study, we have investigated the mechanisms regulating TC21 activation by RasGRF family GEFs. We show that RasGRF GEFs display similar spatial specificities toward TC21 and Ras. We also demonstrate that RasGRFs are capable of activating TC21 by a mechanism regulated by the isoprenoid attached to the GTPase and, unlike Ras, are dependent on an integral PH1 domain. Overall, our results disclose divergences and similarities on the structural and spatial determinants regulating RasGRF GEFs activity toward closely related GTPases.

MATERIALS AND METHODS

Plasmids

Plasmids encoding RasGRF1 mutants have been described previously (Buchsbaum *et al.*, 1996; Freshney *et al.*, 1997; Arozarena *et al.*, 2000), those for RasGRF2 mutants were provided by M. F. Moran (University of Toronto; de Hoog *et al.*, 2000). pCEFL-HA-TC21 and pCEFL-HA-R-Ras were generated by subcloning in-frame wild-type TC21 and wild-type R-Ras in pCEFL-HA. HA-TC21 F204L and F204S were prepared by polymerase chain reaction (PCR)-directed mutagenesis and verified by sequencing. pCEFL targeting vectors harboring the tethering signals: M1, LCK, KDELR, and CD8 α have been described previously (Arozarena *et al.*, 2004; Matallanas *et al.*, 2006). pCEFL-CAV caveola-tethering vector was generated by cloning nucleotides 252-538 of human caveolin-1 in pCEFL. To generate TC21 site-specific vectors, HA-TC21 C199S was prepared by PCR-directed mutagenesis. It was then subcloned in-frame into the targeting vectors. Sequences of the oligonucleotides used are available upon request. pCEFL-FLAG-Cdc42 was provided by Y. Zheng (University of Cincinnati). HA-Cdc42-GAP by R. Cerione. Human RasGRF2 small interfering RNA (siRNA) and control siRNAs were from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a concentration of 25 nM.

Cell Culture

COS-7 and HeLa cells were grown in DMEM supplemented with 10% fetal calf serum. For biochemical analyses, subconfluent COS-7 and HeLa cells were transfected with DEAE-dextran (Arozarena *et al.*, 2000) and with Lipofectamine (Invitrogen, Carlsbad, CA), respectively. For immunofluorescence studies, COS-7 and HeLa cells were transfected with FuGENE transfection reagent (Roche Diagnostics, Mannheim, Germany). Before stimulation, cells were starved for 18 h, unless otherwise indicated. Lysophosphatidic acid (LPA) and ionomycin were from Sigma-Aldrich (St. Louis, MO).

Antibodies

Mouse and rabbit monoclonal anti-hemagglutinin (HA), rabbit polyclonal anti-H-Ras, -early endosome antigen (EEA)1, -panRasGRF, -Sos1, and -extracellular signal-regulated kinase (ERK)2 were from Santa Cruz Biotechnology. Rabbit polyclonal anti-TC21 was supplied by X. R. Bustelo (University of Salamanca). Mouse polyclonal anti-protein disulfide isomerase (PDI) was from Assay Designs (Ann Arbor, MI). Anti-Giantin mouse monoclonal antibodies were supplied by G. Egea (University of Barcelona). Mouse monoclonal anti-transferrin receptor (TFR) was from Zymed Laboratories (South San Francisco, CA). Rabbit and mouse monoclonal anti-caveolin-1, mouse monoclonal anti-TGN38, -tubulin- α , -vinculin, and -paxillin were from BD Biosciences (San Jose, CA). Mouse monoclonal anti-FLAG was from Sigma-Aldrich.

Ras-GTP Loading Assays

Ras-GTP loading assays were performed as described previously (Arozarena *et al.*, 2001). H-Ras-GTP, TC21-GTP, and R-Ras-GTP were affinity sequestered by using glutathione transferase (GST)-Raf-RBD. Immunoblots were performed with anti-HA antibody and quantified by densitometry using Image

1.60 (National Institutes of Health, Bethesda, MD). Activation levels were related to total protein levels as determined by anti-HA immunoblotting in the corresponding total lysates. Statistical analyses were performed using GraphPad software (GraphPad Software, San Diego, CA).

Immunoblotting

Samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose filters as described previously (Ajenjo *et al.*, 2000). Immunocomplexes were visualized by enhanced chemiluminescence detection (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA).

Confocal Immunofluorescence

Cultured cells were washed twice in phosphate-buffered saline (PBS), fixed with ice-cold 3.7% formaldehyde in PBS for 10 min, and washed with cold PBS. They were rinsed in PBS-0.05% Tween 20 (Sigma-Aldrich), incubated for 1 h with the primary antibodies or cholera toxin-fluorescein isothiocyanate (FITC) (Sigma-Aldrich), washed, and incubated for 45 min with the appropriate secondary antibodies conjugated to FITC or Texas Red. Coverslips were mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA), and sealed. Confocal microscopy was performed with an LSM510 microscope (Carl Zeiss, Thornwood, NY), by using excitation wavelengths of 488 nm (for FITC) and 543 nm (for Texas Red).

Sucrose Gradients

Cells were collected and treated as described previously (Matallanas *et al.*, 2006). In brief, cells were collected and resuspended in 25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.25% Triton X-100. Lysates were set at a sucrose concentration of 42.5%. Layers of 3.4 ml of 35% sucrose and 1 ml of 16% sucrose were sequentially overlaid and centrifuged for 18 h at 35,000 rpm. Twelve 0.5-ml fractions were collected and precipitated in 6.5% trichloroacetic acid, resuspended in loading buffer, and fractionated by SDS-PAGE. Nuclei and heavy endomembranes such as the endoplasmic reticulum (ER) and Golgi, remain in the uncollected pellet.

Subcellular Fractionation

Performed in 20 mM HEPES, pH 7.4, buffer, basically as described previously (Freshney *et al.*, 1997).

Statistical Analyses

All statistical data were analyzed and compared for statistically significant differences by Student's *t* test (GraphPad Software).

RESULTS

Characterization of the Subcellular Distribution of TC21

As a first step to understand the spatial regulation of TC21 activation, we studied its subcellular distribution. Although it has been described that overexpressed TC21 localizes in the ER and the Golgi complex (Ohba *et al.*, 2000), a complete characterization had never been conducted. Initially, we attempted to study endogenous TC21 in HeLa cells. However, neither commercially available nor several house-made anti-TC21 antibodies yielded satisfactory results. HeLa cells immunostained with these antibodies exhibited a highly unspecific, scattered punctuate staining. Similar unsatisfactory results were obtained in other cells such as COS-7, BHK, and MCF-7, in which TC21 is expressed at high levels as ascertained by immunoblotting (data not shown). As a consequence, we resorted to transient transfections with minimal amounts of plasmid encoding for HA-TC21. Such "minimal amount" was decided by extrapolating the data obtained for H-Ras, cloned in the same expression vector (pCEFL). In this case, the subcellular distribution of the exogenous protein can be compared with that of the endogenous, and we could determine the amounts of plasmid for which spillage was not detected (data not shown). Remarkably, even when transfecting high quantities of HA-TC21, spillage of the protein was rare. Less than 5% of the cells showed an abnormal or mislocalized distribution when transfected with 50 ng of DNA. The percentage increased just up to 10% when transfected with 500 ng (Supplemental

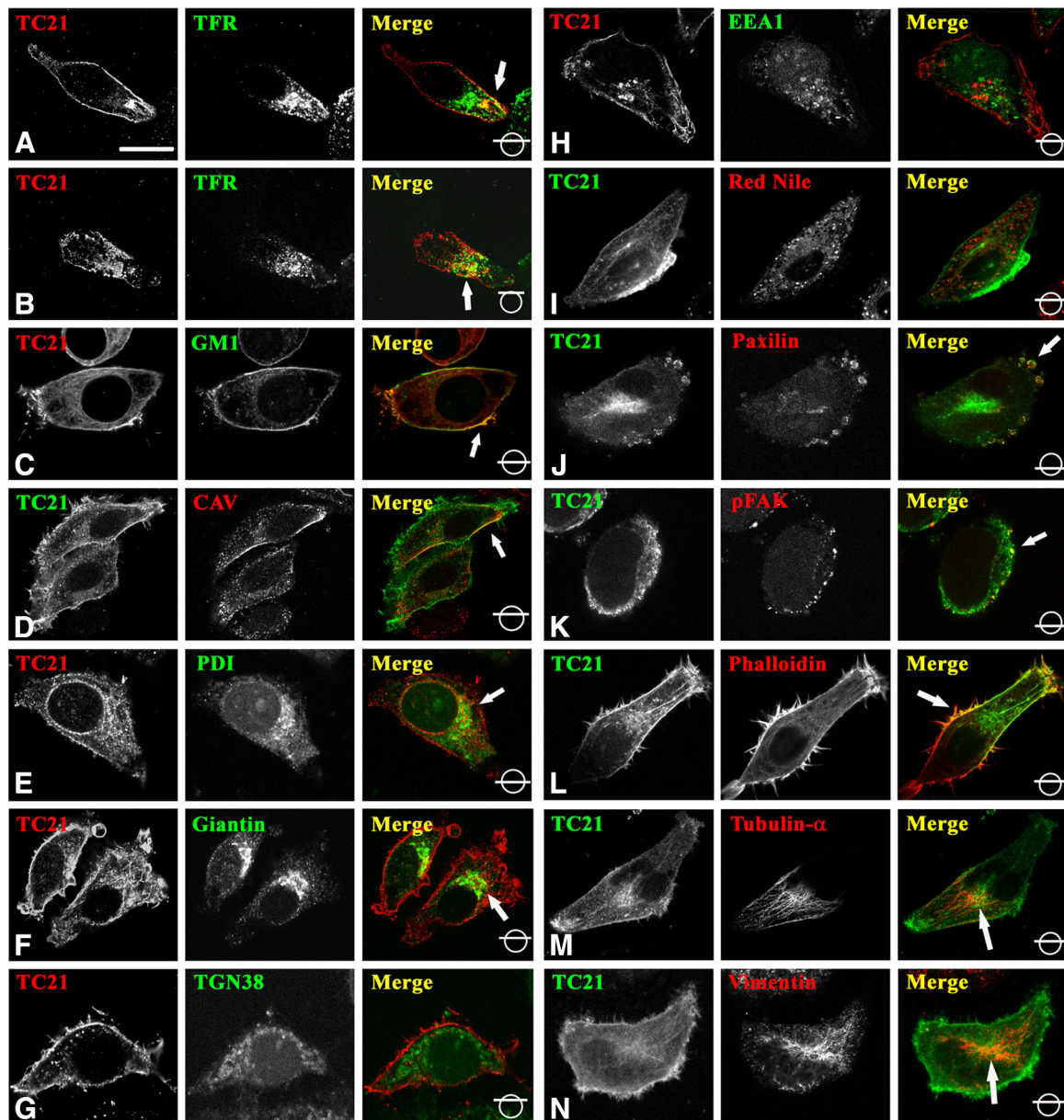


Figure 1. Subcellular localization of TC21. HeLa cells were transfected with HA-TC21 (100 ng) and its colocalization with site-specific markers was analyzed by immunofluorescence. (A–N) Representative confocal micrographs of cells doubly immunostained with anti-HA and anti-TFR (A and B), FITC-cholera toxin (C), anti-caveolin-1 (D), anti-PDI (E), anti-Giantin (F), anti-TGN38 (G), anti-EEA1 (H), Red Nile (I), anti-paxillin (J), anti-phospho-FAK Y397 (K), phalloidin-Texas Red (L), anti-tubulin- α (M), and anti-vimentin (N). Intense colocalization is shown by arrows. The symbol in the bottom right corner indicates the confocal plane displayed. Bar, 10 μ m.

Figure 1A). Immunostaining HeLa cells with anti-HA revealed that TC21 displayed mostly a peripheral localization, with some high-intensity signal at the perinuclear region and some punctuate cytoplasmic associations (Figure 1, left). To identify these structures, we performed double immunofluorescences with specific cellular markers. The TFR, a disordered membrane marker (Vivien *et al.*, 1993), disclosed that TC21 was present at these microdomains at the plasma membrane (PM) and in internal structures, probably corresponding to recycling endosomes derived from this type of membrane (Figure 1, A and B), in which TFR has been observed previously (Mayor *et al.*, 1998). To study the possible distribution of TC21 in lipid raft regions of the PM, we stained with cholera toxin-FITC that recognizes the raft

marker GM1, which exhibited a clear colocalization with TC21 (Figure 1C). Likewise, TC21 also colocalized with the caveolar marker caveolin-1 (Figure 1D). These results demonstrated that TC21 could locate in all types of PM microdomains. Further proof for the localization of TC21 in the different microdomains was provided by membrane fractionation experiments, in which cells were solubilized in low concentrations of Triton X-100 and fractionated in sucrose gradients. It was found that both endogenous and overexpressed TC21 fractionated similarly independently of the cell type, occurring both in the lighter fractions with the raft marker caveolin-1 and in disordered membrane fractions, characterized by the presence of TFR (Supplemental Figure 1B). Continuing with the identification of TC21 localizations,

we monitored its distribution in endomembrane systems. We observed slight colocalization between TC21 and the ER marker PDI at the perinuclear region (Figure 1E), in which it also merged with the *cis*-Golgi marker Giantin (Figure 1F). In contrast, there was no colocalization with the *trans*-Golgi protein TGN38 (Figure 1G), or with the early endosomal marker EEA1 (Figure 1H), nor did TC21 locate in late endosomes, as detected with Red Nile staining (Figure 1I).

It has been reported that R-Ras associates with cytoskeletal structures such as focal adhesions (Furuhjelm and Peranen, 2003), a process determined by its C-terminal domain (Wang *et al.*, 2000). Because TC21 possesses motifs similar to those in R-Ras linked to the formation of focal adhesions (Clark *et al.*, 1996), we analyzed whether it could locate in them. We observed a clear colocalization of TC21 with paxillin and with phosphorylated focal adhesion kinase (FAK), present at these structures (Pendas-Franco *et al.*, 2007), indicative of its association with focal adhesions (Figure 1, J and K). TC21 also strongly colocalized with cortical actin at the cell periphery (Figure 1L), as observed previously for R-Ras (Holly *et al.*, 2005; Jeong *et al.*, 2005). In contrast, the internal cytoskeletal distribution of TC21 corresponded with tubulin- α - and vimentin-positive structures, indicative of its association with microtubules and intermediate filaments (Figure 1, M and N). Overall, these results demonstrated the widespread distribution of TC21 at various cellular structures and organelles.

RasGRF GEFs Activate TC21 at the Plasma Membrane and at the Endoplasmic Reticulum

Once we corroborated that TC21 was present at different localizations, we aimed to analyze whether RasGRF GEFs activity was restricted to specific regions within the cell. For that purpose, we generated TC21 constructs that specifically targeted the GTPase to different membrane platforms. Following the strategy that we had used previously for Ras (Arozarena *et al.*, 2004; Matallanas *et al.*, 2006), we generated an unpalmitoylable mutant, TC21 C199S. To this mutant, we added at its N terminus the cues that would specifically deliver it to ER (M1 BBV protein), lipid rafts (LCK myristoylation signal), Golgi complex (KDELr N193D), and disordered membrane (CD8 α transmembrane domain). All these targeting signals have been successfully used before for Ras (Matallanas *et al.*, 2006). An HA tag was also included to enable the detection of the expressed proteins. We desired to analyze TC21 activation also at caveolae. To this end, we generated CAV-TC21, which harbors at its N-terminus nucleotides 252-538 of human caveolin-1, containing the transmembrane, oligomerization, and scaffolding domains, required to localize it at caveolae (Song *et al.*, 1997) (Supplemental Figure 2). All these constructs were transiently expressed in COS-7 cells and their correct distribution was ascertained by anti-HA immunofluorescence (Figure 2A). To verify beyond doubt that the targeted TC21 proteins were correctly localized, we also performed fractionations in sucrose gradients. As shown previously for endogenous TC21, overexpressed wild-type TC21 fractionated in raft and nonraft fractions, whereas LCK- and CAV-TC21 only fractionated in the lighter fractions and CD8-TC21 was only detected in the denser fractions, indicative of their correct localization (Figure 2B). Neither M1- nor KDELr-TC21 could be detected on either lipid raft or disordered membrane fractions, demonstrating the lack of contamination of peripheral membranes with endomembrane fragments (data not shown). All these results pointed to the correct localization of our targeted TC21 proteins.

Previous results from our group showed that RasGRF1/2 activated H-Ras at the PM and the ER but not at the Golgi complex (Arozarena *et al.*, 2004). It was important to corroborate whether this spatial selectivity was GTPase-specific or whether it applied to all RasGRFs cognate GTPases. For this purpose, we cotransfected the tethered TC21 constructs with RasGRF1 and RasGRF2 in COS-7 cells, which do not express these GEFs endogenously. TC21 activation was analyzed by pull-down assays by using GST-Raf-RBD to affinity precipitate GTP-bound TC21 (Taylor *et al.*, 2001). We observed that both GEFs could induce exchange on TC21 in all of the localizations studied, except for the Golgi complex, in which RasGRP1 could effectively activate it, as observed previously for H-Ras (Bivona *et al.*, 2003; Caloca *et al.*, 2003) (Figure 2C). RasGRF2 was less efficient than RasGRF1 in activating TC21 at the peripheral membrane. This effect was more evident on lipid raft-tethered TC21 than over disordered membrane and caveolar TC21. Conversely, both GEFs displayed similar potentials over ER-tethered M1-TC21 (Figure 2C). These results demonstrated that RasGRF GEFs show similar spatial selectivity toward their cognate GTPases Ras and TC21.

RasGRF2 Activates Farnesylated but Not Geranylgeranylated TC21

Because TC21 can be both farnesylated and geranylgeranylated (Carboni *et al.*, 1995; Roskoski and Ritchie, 1998; Reid *et al.*, 2004), we asked whether such difference on its posttranslational modification could affect its activation by RasGRF GEFs. As a first step, we compared the abilities of the different types of Ras GEFs to stimulate nucleotide exchange on wild-type TC21. For this, we transfected COS-7 cells with HA-TC21 plus the different GEFs, and TC21 activation was analyzed. We observed that RasGRF2 induced nucleotide exchange on TC21 to a lesser extent than RasGRF1 or RasGRP1 (Figure 3A). In this cellular system, Sos1 showed a minimal catalytic activity over TC21, contrary to what has been observed previously in human embryonic kidney 293 cells (Ohba *et al.*, 2000). For comparative reasons, we ascertained that these GEFs induced nucleotide exchange on H-Ras to similar extents (Figure 3A, middle), whereas over R-Ras, only RasGRF1 and RasGRP1 could induce GDP/GTP exchange (Figure 3A, bottom), in complete agreement with previous reports (Gotoh *et al.*, 1997; Ohba *et al.*, 2000).

It has been reported that RasGRF2 is incapable of activating R-Ras due to the modification of this GTPase by a geranylgeranyl group (Gotoh *et al.*, 2001). Because TC21 can be modified by either farnesyl-transferases or geranylgeranyl-transferases, it was possible that RasGRF2 low activity toward TC21 was a consequence of a predominant geranylgeranylation. To assess this hypothesis, we generated TC21 mutants modified in their X amino acid at the CAAX box that directs the posttranslational modification. Thus, if X is a serine, TC21 F204S, it would be modified by a farnesyl-transferase, whereas if it is a leucine, TC21 F204L, a geranylgeranyl group would be added (Reid *et al.*, 2004). Using these mutants, we observed that farnesylated TC21 F204S could be activated pronouncedly by RasGRF2, whereas the geranylgeranylated mutant TC21 F204L was unresponsive to this GEF (Figure 3B). In contrast, RasGRF1 displayed no selectivity toward either form of TC21. These results demonstrated that RasGRF2 exchange activity over TC21 displays specificity toward its farnesylated but not its geranylgeranylated form.

The selectivity of RasGRF2 toward TC21 was further analyzed in HeLa cells, which endogenously express this GEF (Arozarena *et al.*, 2004). In these, overexpression of RasGRF2 results in an enhanced activation of TC21 even under basal conditions (Figure 4A). RasGRF GEFs are distinctively acti-

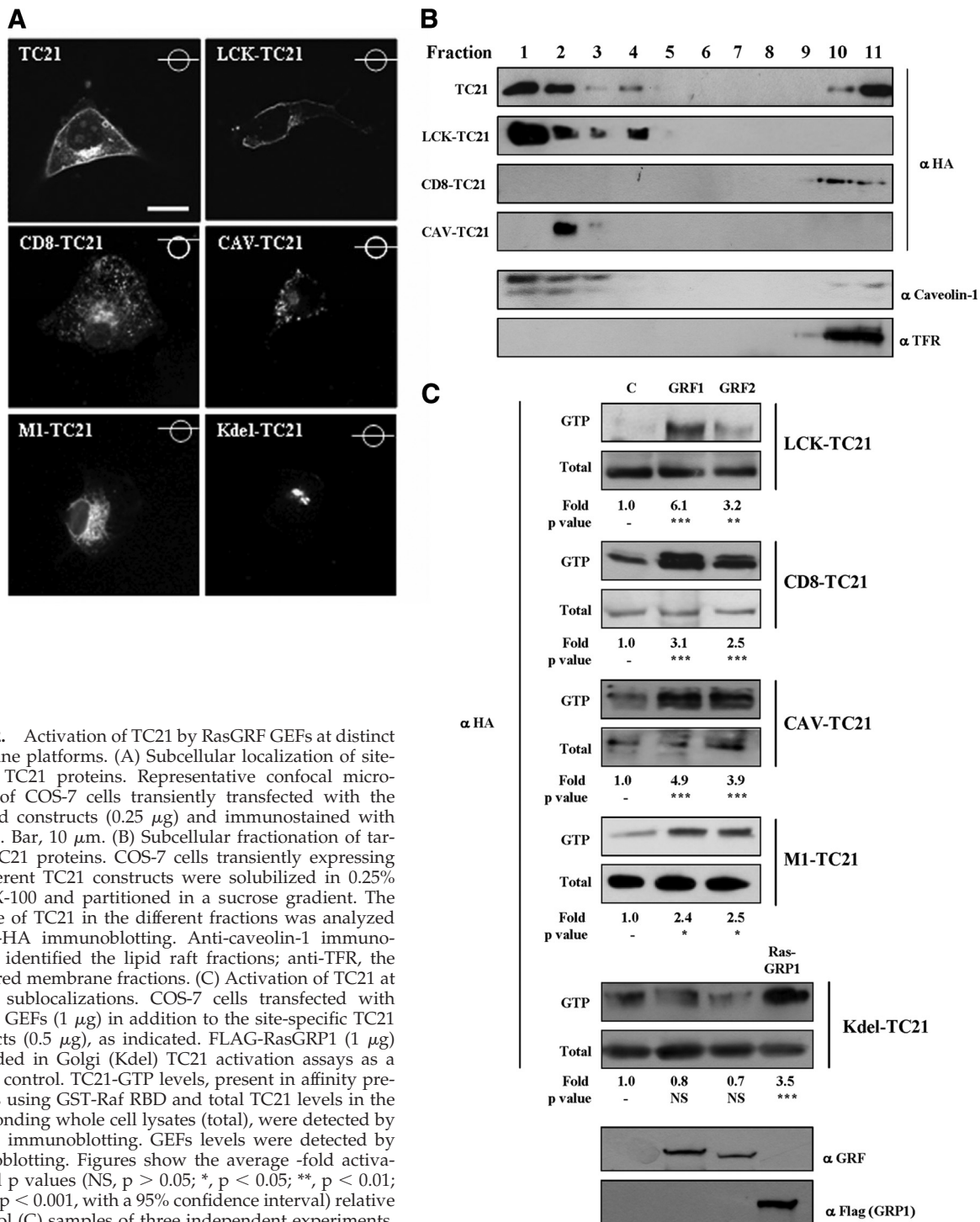


Figure 2. Activation of TC21 by RasGRF GEFs at distinct membrane platforms. (A) Subcellular localization of site-specific TC21 proteins. Representative confocal micrographs of COS-7 cells transiently transfected with the indicated constructs (0.25 μ g) and immunostained with anti-HA. Bar, 10 μ m. (B) Subcellular fractionation of targeted TC21 proteins. COS-7 cells transiently expressing the different TC21 constructs were solubilized in 0.25% Triton X-100 and partitioned in a sucrose gradient. The presence of TC21 in the different fractions was analyzed by anti-HA immunoblotting. Anti-caveolin-1 immunoblotting identified the lipid raft fractions; anti-TFR, the disordered membrane fractions. (C) Activation of TC21 at defined sublocalizations. COS-7 cells transfected with RasGRF GEFs (1 μ g) in addition to the site-specific TC21 constructs (0.5 μ g), as indicated. FLAG-RasGRP1 (1 μ g) was added in Golgi (Kdel) TC21 activation assays as a positive control. TC21-GTP levels, present in affinity precipitates using GST-Raf RBD and total TC21 levels in the corresponding whole cell lysates (total), were detected by anti-HA immunoblotting. GEFs levels were detected by immunoblotting. Figures show the average -fold activation and p values (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, with a 95% confidence interval) relative to control (C) samples of three independent experiments.

vated by stimuli mediated by G protein-coupled receptors, such as LPA, and by calcium ionophores, like ionomycin (Quilliam *et al.*, 2002). In response to these, TC21 was markedly activated, and this activation was amply diminished when the expression of endogenous RasGRF2 was repressed using a siRNA or after transfection of a dominant-negative form of the GEF, lacking the Cdc25 domain (Figure 4A). Similarly, the selectivity for farnesylated TC21 was also observed for endogenous RasGRF2: LPA induced a significant activation of TC21 F204S, which was inhibited when endogenous Ras-

GRF2 was down-regulated either by the siRNA or by the dominant-negative. By contrast, TC21 F204L was unresponsive to LPA (Figure 4B). Similar results were obtained in Jurkat cells, another cellular system that endogenously express RasGRF2 (Ruiz *et al.*, 2007) (data not shown).

Subcellular Distribution of TC21 Is Not Dependent on Its Isoprenoid Modification

The profound differences on the activity that RasGRF2 displayed toward geranylgeranylated and farnesylated TC21

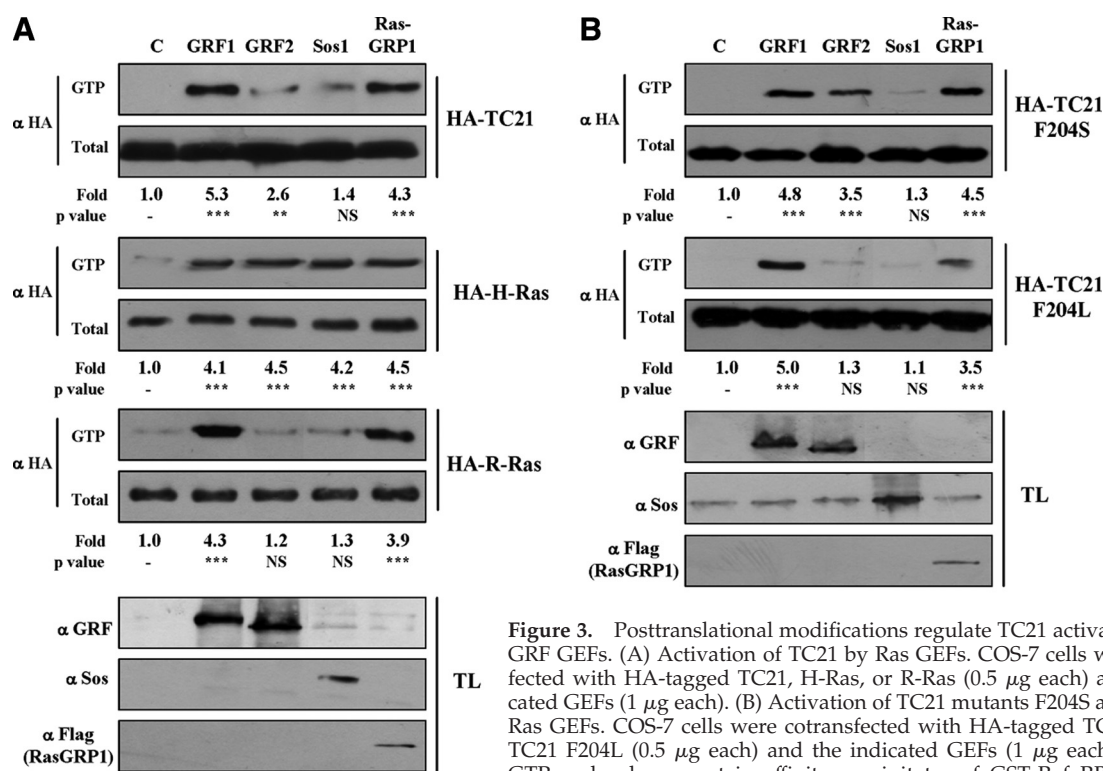


Figure 3. Posttranslational modifications regulate TC21 activation by Ras-GRF GEFs. (A) Activation of TC21 by Ras GEFs. COS-7 cells were cotransfected with HA-tagged TC21, H-Ras, or R-Ras (0.5 μ g each) and the indicated GEFs (1 μ g each). (B) Activation of TC21 mutants F204S and F204L by Ras GEFs. COS-7 cells were cotransfected with HA-tagged TC21 F204S or TC21 F204L (0.5 μ g each) and the indicated GEFs (1 μ g each). Activated GTPase levels present in affinity precipitates of GST-Raf RBD and total

GTPase levels in the corresponding whole cell lysates (total) were detected by anti-HA immunoblotting. GEFs levels were detected by immunoblotting. Figures show the average -fold activation and p value (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, with a 95% confidence interval) relative to control (C) samples of three independent experiments.

could be a consequence of alterations in the subcellular localization of this GTPase depending on the prevailing posttranslational modification. Analyzing the localization of the different forms of TC21 ectopically expressed in HeLa cells, we could not observe major differences between the geranylgeranylated and farnesylated TC21 forms or between either form and the wild-type GTPase (Figure 5A). These similar distribution patterns were corroborated in other settings such as COS-7 cells (Supplemental Figure 3). The distribution in PM microdomains was further investigated by membrane fractionation. In an identical manner to endogenous TC21 (Supplemental Figure 1), HA-TC21 was present both in cholesterol-rich (lipid rafts/caveolae) and in bulk membrane fractions. The mutant TC21 F204L presented a similar distribution to wild-type TC21, the same as TC21 F204S, even though it was slightly more enriched in disordered membrane fractions (Figure 5B). Furthermore, by colocalization immunofluorescence we observed that the different isoprenoid modifications did not alter the degree of colocalization of TC21 with RasGRF1 and RasGRF2 neither under basal conditions, nor under stimulation with LPA or ionomycin (Figure 6 and Supplemental Figure 4). These data concluded that the different TC21 posttranslational modifications do not significantly alter its subcellular localization and, subsequently, RasGRF2 selectivity toward farnesylated TC21 is not related to its localization.

The PH1 Domain of RasGRF GEFs Is Essential for TC21 Activation

It was of interest to determine which domains in RasGRF GEFs were implicated in the activation of TC21 and whether such domains were the same domains involved in the activation of Ras. For this purpose, we used deletion mutants

for RasGRF1 (Arozarena *et al.*, 2000) and RasGRF2 (de Hoog *et al.*, 2000) used previously in similar studies on Ras activation (Figure 7A). In addition, in the case of RasGRF1, we also used three point mutants: PH1⁻, DH⁻, and IQ⁻, that contain point mutations that diminish the functionality of the respective domain without affecting the global structure of the protein (Buchsbaum *et al.*, 1996; Freshney *et al.*, 1997).

Analyzing the activation of TC21 induced by RasGRF1 domain deletion mutants, we observed that deletion of either the PH1 or DH domains clearly affected the catalytic potential of Ras-GRF1. Similar results were obtained when we looked at the activation of R-Ras activation (Figure 7B). However, as demonstrated previously (Arozarena *et al.*, 2000), this was not the case for H-Ras, which was only sensitive to the deletion of the DH domain. These results were ratified when we used the point mutants for the PH1 and DH domains, ruling out gross structural changes as a possible cause (Figure 7C). In the case of RasGRF2, activation of TC21 was also dependent on the integrity of the PH1 domain. Similarly to what has been reported previously for H-Ras activation (de Hoog *et al.*, 2000) and in contrast to RasGRF1, RasGRF2 Δ DH maintained its exchange potential over TC21 (Figure 7D).

It was of importance to determine whether RasGRF GEFs used the same structural domains for inducing exchange on TC21 subject to different isoprenoid modifications. To this end, we studied the catalytic potential of RasGRF1/2 deletion mutants over TC21 F204S, farnesylated, and F204L, geranylgeranylated, variants. Interestingly, the PH1 domain was fully dispensable for the activation of farnesylated TC21 F204S by both RasGRF1 and RasGRF2. In contrast, both GEFs required their PH1 domain for inducing exchange on geranylgeranylated TC21 F204L (Figure 7E). Overall, our

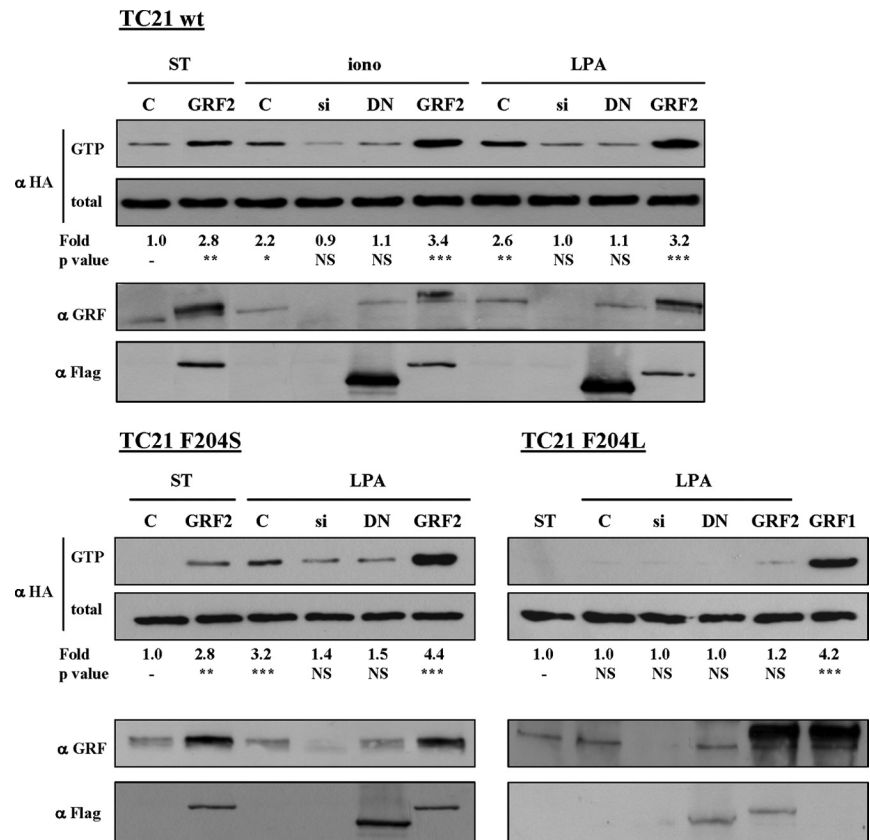


Figure 4. Agonist-induced activation of TC21 regulated by endogenous RasGRF2. Activation of TC21 in HeLa cells in response to LPA and Ionomycin. (upper panels) HeLa cells were transfected with HA-TC21 (1 μ g). Where indicated, the concentration of RasGRF2 was increased by transfection with FLAG-RasGRF2 (1 μ g) (GRF2) or repressed by an anti-RasGRF2 siRNA (si). Endogenous RasGRF2 was inhibited by transfecting FLAG-RasGRF2 Δ cdc25 (1 μ g) (DN). (lower panels) Activation of HA-TC21 F204S and HA-TC21 F204L, as described above. For TC21 F204L, RasGRF1 (1 μ g) was used as a positive control. In all cases, cells were starved for 18 h and stimulated for 5 min with LPA (10 μ M) or ionomycin (1 μ M). Activated GTPase levels present in affinity precipitates using GST-Raf RBD and total GTPase levels in the corresponding whole cell lysates (total) were detected by anti-HA immunoblotting. GEFs levels were detected by immunoblotting. Figures show the average -fold activation and p values (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, with a 95% confidence interval) relative to control (C)/ST samples of three independent experiments.

results demonstrate that RasGRF GEFs structural domain requirements for the activation of R-Ras GTPases are different to those for H-Ras, as a consequence of the particular isoprenoid modification of this subfamily of GTPases.

LPA- and Ionomycin-induced Activation of TC21 Mediated by RasGRF1 and RasGRF2

The responsiveness of RasGRF GEFs to external stimuli, at least in H-Ras activation, is dependent on distinct N-terminal domains, both for ionomycin (Buchsbaum *et al.*, 1996; Freshney *et al.*, 1997; Fan *et al.*, 1998; de Hoog *et al.*, 2000; Arozarena *et al.*, 2004) and for LPA (Innocenti *et al.*, 1999; Arozarena *et al.*, 2004). As such, we investigated whether ionomycin- and LPA-induced potentiation of RasGRF activity toward TC21 required the same structural domains. To do so, COS-7 cells were cotransfected with suboptimal concentrations of RasGRF1 or RasGRF2 plus HA-TC21, and, after starvation, TC21 activation was assayed after stimulation. As shown in Figure 8A, at these concentrations neither RasGRF1 nor RasGRF2 affected TC21 activity. However, for RasGRF1, both LPA and ionomycin induced an increase in TC21-GTP levels in the presence of the wild-type GEF, but the induction was remarkably lower when any of the deletion mutants was the mediator. In RasGRF2, all the deletion mutants lost their response to ionomycin to great extents, with the exception of Δ DH that maintained its TC21-activating capacity. To a lesser extent, these deletion mutants also displayed a diminished ability to respond to LPA, in particular Δ IQ (Figure 8B).

In this same context, we also investigated whether some correlation between the capacity to mediate TC21 nucleotide exchange and the degree of localization to membranes existed for the different deletion mutants. Using a simple frac-

tation protocol, we ascertained the particulate and cytosolic content of the RasGRF GEFs mutant forms, under starvation and upon stimulation with LPA or ionomycin. In general, we did not observe any remarkable change in the membranous fraction-bound content for any of the mutant forms after agonist treatment, which clearly indicated that their nucleotide exchange potential is not related to their ability to associate to membranes (Figure 8C). Interestingly, RasGRF1 and RasGRF2 Δ DH mutants showed the same distribution, with practically no particulate content, despite that their abilities to induce GDP/GTP exchange over Ras and TC21 are completely different. These results demonstrated that the exchange potential of RasGRF GEFs is not dependent on its stable association to membranes.

Cdc42 Regulates TC21 Activation by RasGRF GEFs

Past results from our laboratory demonstrated that RasGRF exchange activity toward H-Ras is negatively regulated by Cdc42 when GDP-bound (Arozarena *et al.*, 2000, 2001). Therefore, it was of interest to determine whether this was also applicable to the activation of TC21. For this purpose, we used Cdc42-GAP that when overexpressed retains Cdc42 in its GDP-bound form (Leonard *et al.*, 1998). To begin with, we used HeLa cells, which harbor endogenous RasGRF2. As shown in Figure 4A, in these cells both LPA and ionomycin induced a potent TC21 activation, inhibitable by down-regulation of RasGRF2 levels by an siRNA. The transfection of Cdc42-GAP yielded similar effects as RasGRF2 down-regulation: a pronounced reduction of TC21 activation (Figure 9A). To further substantiate this observation, we resorted to our ectopic expression system using COS-7 cells. We found that TC21 activation as induced by transfected RasGRF1 or RasGRF2 was largely unaffected by cotransfected

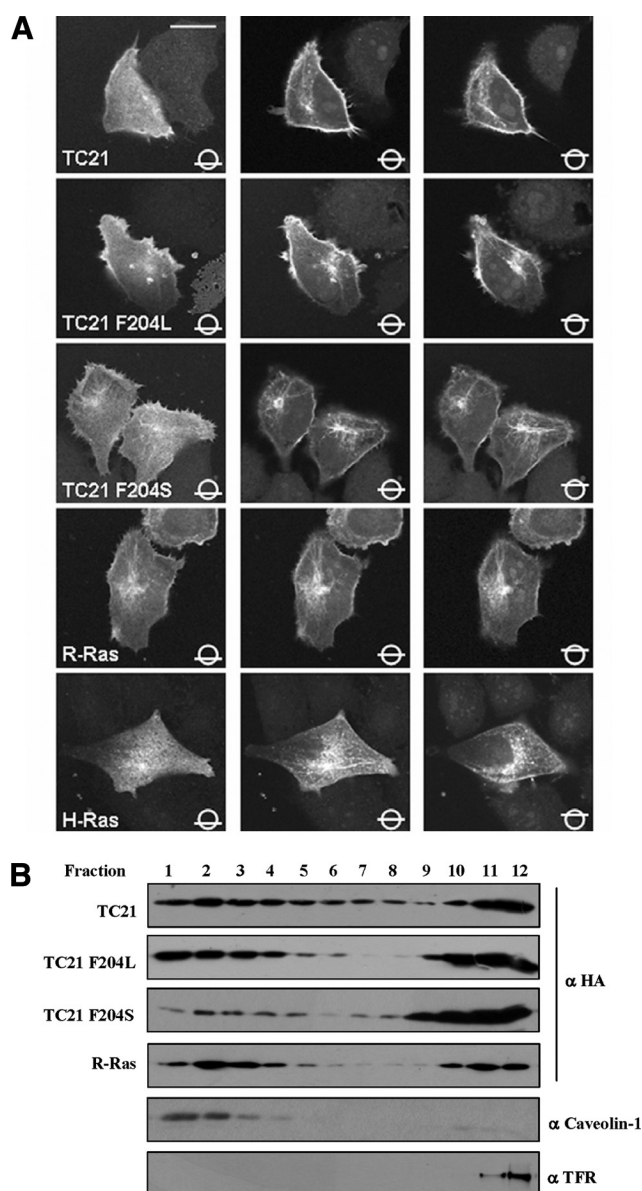


Figure 5. Subcellular distribution of the distinct TC21 forms in HeLa cells. (A) Representative confocal micrographs of HeLa cells transiently transfected with HA-TC21, HA-TC21 F204L, HA-TC21 F204S, HA-R-Ras and HA-H-Ras (0.25 μ g each). The distribution of the different proteins was detected by specific immunostaining using anti-HA antibodies. Three different confocal sections are represented in each case. (B) TC21 distribution in PM microdomains. HeLa cells transiently expressing the different GTPases were solubilized in 0.25% Triton X-100 and partitioned in sucrose gradients. The presence of the ectopic proteins in the different fractions was analyzed by anti-HA immunoblotting. Anti-caveolin-1 immunoblotting identified the lipid raft fractions, and anti-TFR identified the disordered membrane fractions.

Cdc42-GAP, probably because not sufficient endogenous Cdc42 is available to counteract the effects of high levels of RasGRFs. This notion was made clear when wild-type Cdc42 was transfected in addition to Cdc42-GAP. In this case, Ras-GRFs-induced TC21 activation was almost abolished (Figure 9B, top). As reported previously (Arozarena *et al.*, 2000), exogenous Cdc42 per se could inhibit TC21 acti-

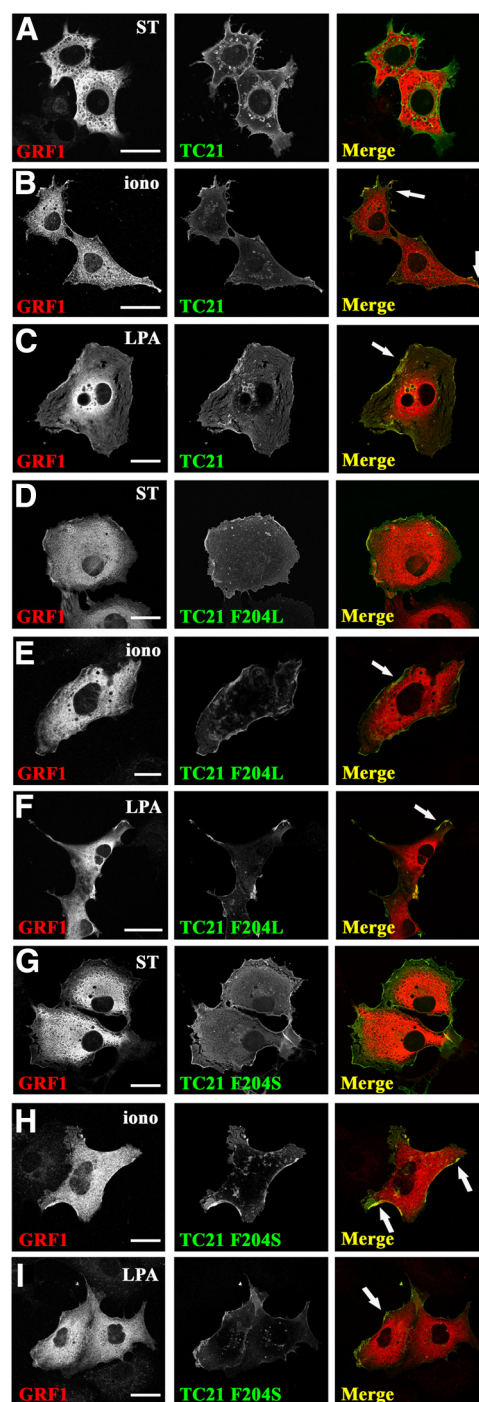


Figure 6. Colocalization of RasGRF1 with TC21. (A–I) Representative confocal micrographs of COS-7 cells transiently transfected with RasGRF1 (0.5 μ g) plus HA-TC21, wild type, or the mutant forms (0.25 μ g each), as shown. The distribution of the RasGRF proteins was detected by specific immunostaining with anti-GRF antibodies. TC21 was detected with anti-HA antibodies. All micrographs represent equatorial sections at the level of the nucleus. Intense colocalization is shown by arrows. Cells were subjected to 18-h starvation (ST) and stimulated for 5 min with LPA (10 μ M) or ionomycin (1 μ M), as indicated. Bar, 10 μ m.

vation to a significant extent. Similar results were encountered when assaying Ras activation as induced by the Ras-GRF GEFs (Figure 9C, top).

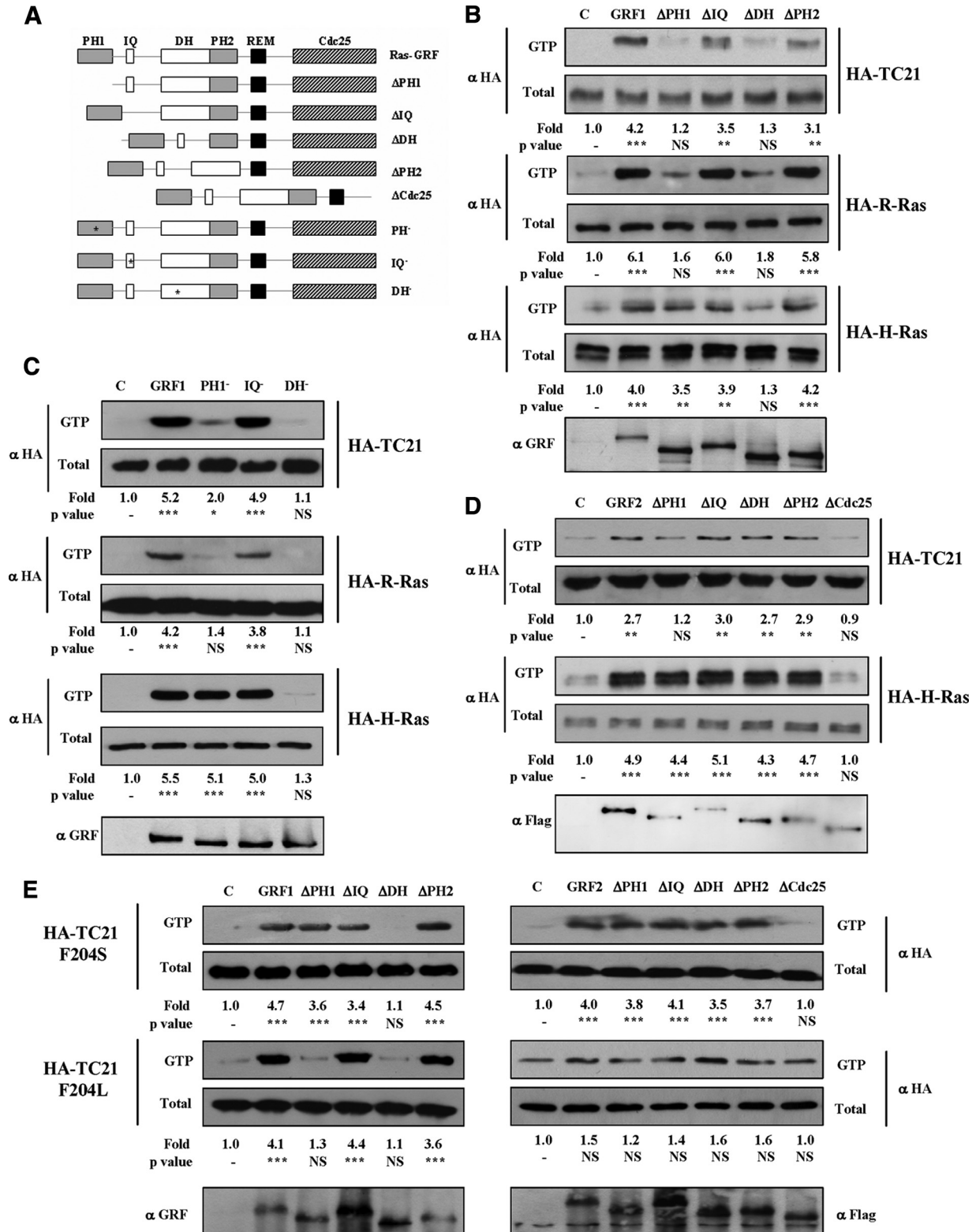


Figure 7. Structural domains requirements for TC21 activation by RasGRF GEFs. (A) Diagram representing the different RasGRF mutants used. (B–D) RasGRF1 deletion mutants (B), point mutants (C), and RasGRF2 mutants (D) were tested in their catalytic potential toward TC21. H-Ras and R-Ras activation also was analyzed where indicated. COS-7 cells were cotransfected with HA-tagged TC21, H-Ras, or R-Ras (0.5 μ g each) and the indicated RasGRF mutants (1 μ g each). GTPase activation was determined as described in *Materials and Methods*. RasGRF and RasGRF2 mutant levels were detected by anti-GRF and anti-FLAG immunoblotting, respectively. (E) Activation of TC21 F204S and F204L mutants by RasGRF1 (left) and RasGRF2 (right) deletion mutants. Figures show the average -fold activation and p values (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, with a 95% confidence interval) relative to control (C) samples of three independent experiments.

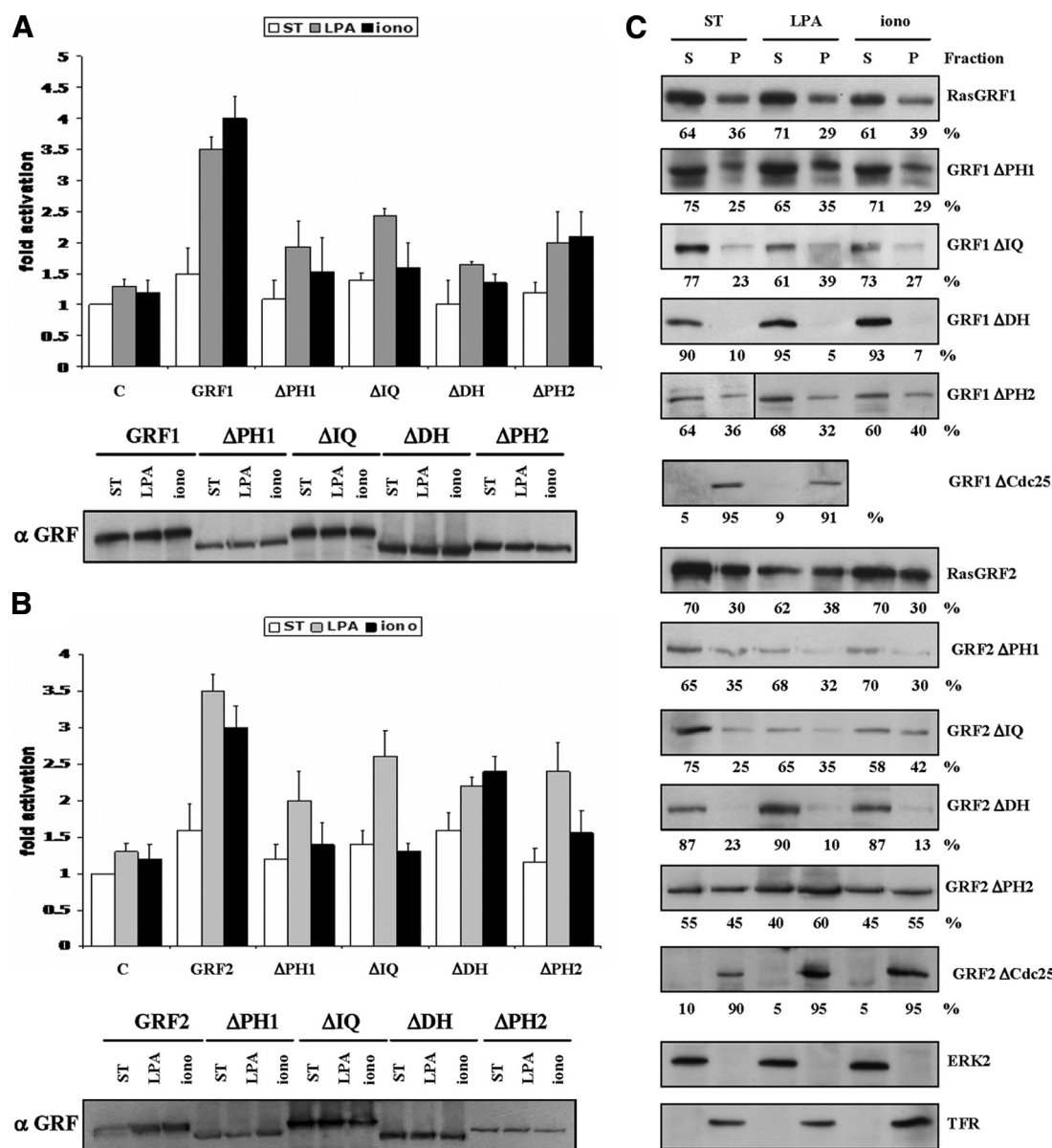


Figure 8. Agonist-induced activation of TC21 mediated by RasGRF GEFs does not correlate with PM translocation. (A and B) Activation of TC21 induced by LPA and ionomycin and mediated by RasGRF1 (A) and RasGRF2 (B) deletion mutants. COS-7 cells were cotransfected with HA-TC21 (0.5 μ g) and suboptimal concentrations (0.1 μ g) of the distinct GEFs. After 18-h starvation, cells were treated with 10 μ M LPA or 1 μ M ionomycin for 5 min. TC21-GTP levels were detected as described previously. Data show mean \pm SEM of three independent experiments, relative to the TC21-GTP levels detected in control cells. Expression levels of the different RasGRF1 and RasGRF2 forms were detected by anti-GRF and anti-FLAG immunoblotting, respectively. (C) Subcellular distribution of RasGRF GEFs under agonist stimulation. COS-7 cells were treated as described above and fractionated in S100 soluble (S) and P100 particulate (P) fractions. The presence of RasGRF was revealed by immunoblotting. ERK2 and TFR immunoblots served as controls for the soluble and particulate fractions. Figures show the percentage of RasGRF present at each fraction, represented as the average of three independent experiments.

Finally, we tested whether Cdc42-GDP could inhibit RasGRF-induced TC21 activation globally or, in contrast, whether its effects were restricted to some of the subcellular localizations where TC21 is present. We explored this possibility by using the previously described TC21-targeted constructs. COS-7 cells were transfected with the site-specific TC21 constructs, the RasGRF GEFs plus Cdc42-GAP and Cdc42 wild type. We observed that the negative effect of Cdc42-GDP over site-specific TC21 activation, as induced by RasGRF1 and RasGRF2, was evident to a significant extent in all the localizations studied (Figure 9B). Similar effects were observed on Ras activation (Figure 8C). These results

suggested that the spatial regulation of RasGRF GEFs by Cdc42-GDP is largely independent of the GTPase being targeted by the GEFs and of the subcellular localization that the cognate GTPases occupy.

DISCUSSION

In this study, we have investigated the mechanisms regulating the activation of TC21 by RasGRF GEFs, paying particular attention to spatial and structural determinants. Regarding subcellular distribution, we have found that TC21 is

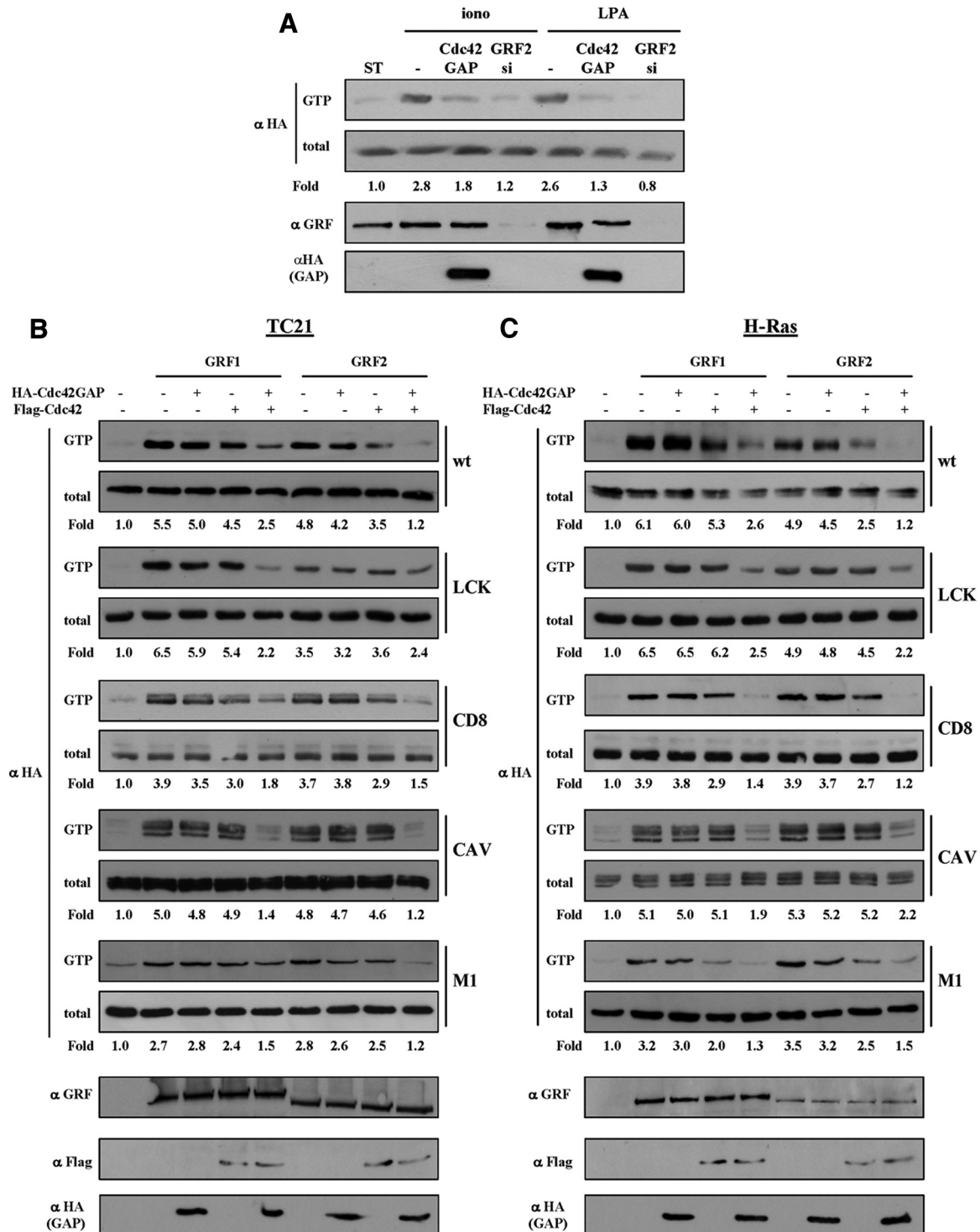


Figure 9. Effects of Cdc42-GDP on TC21 activation induced by RasGRF GEFs. (A) Effects of Cdc42-GDP on agonist-induced TC21 activation in HeLa cells. HeLa cells were transfected with HA-TC21 (1 μ g) and HA-Cdc42-GAP (1 μ g) or an anti-RasGRF2 siRNA (si) where indicated. Cells were starved for 18 h and stimulated for 5 min with LPA (10 μ M) or ionomycin (1 μ M). Activated GTPase levels present in affinity precipitates of GST-Raf RBD and total GTPase levels in the corresponding whole cell lysates (total) were detected by anti-HA immunoblotting. (B) Site-specific effect of Cdc42-GDP on RasGRF-mediated TC21 activation. COS-7 cells were transfected with RasGRF GEFs (1 μ g), as indicated, in addition to HA-TC21 or site-specific HA-TC21 constructs (0.5 μ g), in addition to empty vector (–), 0.5 μ g of FLAG-Cdc42 (+), and 0.5 μ g of HA-Cdc42-GAP (+), as indicated. TC21 activation was determined as described in *Materials and Methods*. RasGRF, Cdc42, and Cdc42-GAP levels were detected by immunoblotting. (C) Site-specific effect of Cdc42-GDP on RasGRF-mediated H-Ras activation. Figures show the average -fold activation relative to control samples of three independent experiments.

widespread in most membrane systems. Previous studies reported TC21 presence at the ER, Golgi (Ohba *et al.*, 2000), and caveolin-rich fractions (Lisanti *et al.*, 1994). At the Golgi, we show that TC21 locates solely in the *cis*-Golgi network. Like other GTPases, such as H-Ras and R-Ras (Choy *et al.*, 1999; Furuhielm and Peranen, 2003), TC21 could use the exocytic route to reach its PM destinations. Interestingly, we did not detect TC21 in early and late endosomes, suggesting that TC21 may not be internalized, at least through the type of endosomes marked by these stains, because it can be found in cytoplasmic vesicles rich in TFR. Further studies are required to clarify this. In addition, we report TC21 association with focal adhesions, in accordance with its known participation in integrin signaling (Keely *et al.*, 1999; Hansen *et al.*, 2003). TC21 also colocalized with cortical actin at the cell periphery, as observed previously for R-Ras (Holly *et al.*, 2005; Jeong *et al.*, 2005). We have also found TC21 colocalizing with microtubules and intermediate filaments, further supporting its role in the regulation of the cytoskeleton and its participation in related functions such as adhesion, migration, and invasion (Clark *et al.*, 1996; Keely *et al.*, 1999; Huang *et al.*, 2004; Arora *et al.*, 2005; Jeong *et al.*, 2005; Pozzi *et al.*, 2006).

We show that RasGRF2 can induce nucleotide exchange on TC21, although to a lesser extent than RasGRF1 or RasGRP1, known previously to activate GTPases of the R-Ras family (Gotoh *et al.*, 1997; Ohba *et al.*, 2000; Quilliam *et al.*, 2002). As shown previously (Gotoh *et al.*, 2001), RasGRF2 was incapable of inducing the activation of R-Ras, probably as a result of the characteristic geranylgeranylation of this GTPase. In a similar manner, RasGRF2 inability to activate TC21 could be a consequence of the predominant geranylgeranylation of TC21 in the cellular system used in our studies. We have confirmed this hypothesis by showing that TC21 F204L, exclusively geranylgeranylated, is refractory to nucleotide exchange in response to RasGRF2, as opposed to the farnesylated F204S mutant, which can be potently activated by this GEF. These observations reinforce the notion that RasGRF2 activity is critically dependent on the post-translational modifications suffered by its cognate GTPases. Although this isoprenoid-dependent regulation was originally described in R-Ras (Gotoh *et al.*, 2001), its physiological relevance was limited, because R-Ras is not naturally farnesylated (Reid *et al.*, 2004), unlike TC21 that can be both farnesylated and geranylgeranylated (Roskoski and Ritchie, 1998; Reid *et al.*, 2004). Thus, in a given tissue, the ratio between GGTs and FTs could exert a regulatory role on signal specificity by dictating the predominant isoprenoid modification in TC21 and thereby its susceptibility to RasGRF2 action. Tissue-specific differences in GGTs/FTs relative activities have been described previously (Morris and Pullarkat, 1995). This study also reported that brain white matter exhibits higher FTase activity than gray matter, suggesting a region-specific susceptibility of TC21 to RasGRF2 in a brain area in which RasGRFs are particularly enriched (Zippel *et al.*, 1997). Another example of such regulation would be RhoB, which can be either farnesylated or geranylgeranylated (Adamson *et al.*, 1992); it suppresses transformation only when geranylgeranylated (Mazieres *et al.*, 2005).

With respect to spatial regulation, our results demonstrate that TC21 activation by RasGRF GEFs occurs in all membrane platforms except for the Golgi complex, with a similar situation reported for H-Ras (Arozarena *et al.*, 2004; Ruiz *et al.*, 2007). Apparently, the isoprenoid modifying TC21 is not relevant for its subcellular localization, because the wild-type form and both F204S and F204L mutants display sim-

ilar distributions. Probably, the distribution of R-Ras GTPases is more dependent on their core C-terminal domains, which in R-Ras determine its association to focal adhesions (Furuhielm and Peranen, 2003), cortical actin filaments (Jeong *et al.*, 2005), and lipid rafts (Hansen *et al.*, 2003). These results suggest that TC21 isoprenoid-dependent susceptibility to activation by RasGRF2 is not, in general, related to changes in spatial distribution but rather, probably depends on structural restraints, such as those described in R-Ras (Gotoh *et al.*, 2001). When analyzing the space-related activation of TC21 elicited by RasGRF GEFs, we observed that it is most prominent at PM microdomains than at endomembranes. Also, RasGRF1 is a better activator in most localizations, with the exception of the ER in which its ability to induce GDP/GTP exchange on TC21 is similar to RasGRF2. The mechanisms underlying in these variations are unknown, although they could be related to RasGRF1 and -2 having different capacities for accessing distinct membrane platforms.

We have characterized the structural domains in RasGRF GEFs necessary for TC21 activation. Interestingly, both RasGRF1 and RasGRF2 require their PH1 domain for inducing exchange on TC21 and R-Ras. More interestingly, this PH1 dependence seems to be restricted to the activation of geranylgeranylated GTPases because it was not observed in H-Ras or farnesylated TC21. One possibility is that the PH1 domain is required for RasGRF GEFs to reach microdomains in which geranylgeranylated GTPases reside. A second possibility is that the PH1 domain somehow mediates in the direct interaction of RasGRF GEFs with geranylgeranylated but not farnesylated GTPases. In addition, RasGRF1 also requires its DH domain for activating TC21. This dependence was not observed for RasGRF2, in spite of an 85% homology between both DH domains (Fam *et al.*, 1997). A similar situation has been reported for H-Ras (Arozarena *et al.*, 2000; de Hoog *et al.*, 2000).

Regarding external agonists, we show that LPA and ionomycin potentiate RasGRF GEFs activity on TC21. Both stimuli have been shown to enhance H-Ras (Farnsworth *et al.*, 1995; Zippel *et al.*, 2000) and R-Ras activation (Gotoh *et al.*, 1997) by RasGRF1. These also induced a significant RasGRF2-mediated activation of TC21, despite its low basal activity toward this GTPase. All RasGRF N-terminal regulatory domains are required to some extent for TC21 agonist-induced activation, similarly to what has been reported for H-Ras (Buchsbaum *et al.*, 1996; Freshney *et al.*, 1997; Fan *et al.*, 1998; Innocenti *et al.*, 1999; de Hoog *et al.*, 2000; Arozarena *et al.*, 2004). Noticeably, we have observed that agonist-induced activation of RasGRFs does not entail gross changes in the direct association of the GEFs to membranes. Furthermore, we have observed that RasGRF functions are not dependent on its stable association to membranes, because Δ PH1 mutants, with low TC21-GEF activity, display a membrane association similar to wild-type proteins, whereas RasGRF1 and RasGRF2 Δ DH mutants, showing opposed activities toward H-Ras and TC21, exhibit an identical distribution, being absent from the particulate fraction.

Finally, we have investigated the role of Cdc42 in the regulation of TC21 activation by RasGRFs. Cdc42 in its GDP-bound form inhibits RasGRF1-mediated activation of H-Ras (Arozarena *et al.*, 2000, 2001; Rabiet *et al.*, 2002). We have observed that, similarly to H-Ras, a Cdc42-GAP diminishes RasGRF1 and RasGRF2 exchange activity on TC21, an effect observable at all of its sublocalizations. Even though the mechanism whereby Cdc42-GDP inhibits RasGRFs functions is still unclear, these results demonstrate that it affects all cognate GTPases and is, to a large extent, localization

independent. In contrast to what was proposed previously, that Cdc42-GDP prevented H-Ras activation by inhibiting RasGRF1 translocation to membranes (Arozarena *et al.*, 2000), here we demonstrate that stable association to membranes is not essential for RasGRFs being functional. Thus, Cdc42 could somehow regulate GEFs access to the proximity of membranes, where the interaction with cognate GTPases would ensue. Further experimentation is required to prove this point.

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